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**A STUDY OF SOME OF THE METABOLIC EFFECTS
OF TOTAL HEPATECTOMY IN THE RAT**



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A STUDY OF SOME OF THE METABOLIC EFFECTS
OF TOTAL HEPATECTOMY IN THE RAT

by

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The present thesis is based on the following papers:

- I. A microsurgical method for total hepatectomy in the rat.
T. Holmin, G. Alinder and P. Herlin.
Accepted for publication in Eur Surg Res.
- II. The influence of total hepatectomy on cerebral energy state, ammonia-related amino acids of the brain and plasma amino acids in the rat.
T. Holmin, C.-D. Agardh, G. Alinder, P. Herlin and B. Hultberg.
Submitted for publication.
- III. Combined or individual administration of branched-chain amino acids following hepatectomy in the rat: Effects on amino acids and indoleamines in brain.
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- IV. Effect of infusing the branched-chain amino acids on concentrations of amino acids in plasma and brain and on brain catecholamines after total hepatectomy in the rat.
J.H. James, P. Herlin, L. Edwards, C.A. Nachbauer and J.E. Fischer.
Life Sciences 30:1361-1368, 1982.
- V. Effect of total hepatectomy and administration of branched chain amino acids on regional norepinephrine, dopamine and amino acids in rat brain.
P. Herlin, J.H. James, C.A. Nachbauer and J.E. Fischer.
Submitted for publication.

In the text the papers are referred to by their Roman numerals.

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HEPATIC ENCEPHALOPATHY

Hepatic encephalopathy may occur in patients with chronic liver disease and spontaneous or surgically induced portosystemic shunting of blood. It is a neuropsychiatric syndrome with cerebral dysfunction and neuromuscular disturbances. The symptoms may vary from mild personality changes to unresponsive coma and are either slowly progressive, resulting in irreversible neurological damage or episodic with full recovery possible from any stage. Acute hepatic coma occurs in association with massive liver necrosis without pre-existing liver disease. This condition is seen in viral hepatitis, drug induced liver damage, mushroom poisoning or fatty degeneration of the liver during pregnancy. Hepatic encephalopathy is associated with several metabolic disturbances and it has been suggested that some of these play a role in the etiology of the syndrome. In the following, ammonia, short-chain fatty acids, mercaptans, the synergistic hypothesis, and the amino acid neurotransmitter theory will be discussed.

Ammonia

Increased concentrations of ammonia in arterial and venous blood are commonly demonstrated in patients with hepatic failure. In some studies high levels of ammonia often correlate with encephalopathy (1, 2). Determination of ammonia in blood is the most usual biochemical test in hepatic encephalopathy. The level of ammonia, however, does not always correlate with the grade of encephalopathy, and some patients with hepatic encephalopathy have normal ammonia concentrations in arterial blood (3).

Patients with liver disease who ingest ammonia-generating substances may develop encephalopathic changes similar to those in patients with impending hepatic coma (4).

Treatment aimed at decreasing blood ammonia levels, i.e. antibiotics, lactulose or reduction of protein intake, may improve encephalopathy (5,6). Ammonia is mainly generated from the gastrointestinal tract, kidneys and skeletal muscle. Ammonia levels in systemic blood are increased in hepatic failure and portosystemic shunting due to shunting of portal blood around the liver and decreased capacity of the liver to detoxify ammonia (7).

Several mechanisms by which ammonia exerts its toxic effects have been proposed. Power failure of the brain because of interference with brain energy metabolism has been suggested as a possible cause of encephalopathy. Thus, Schenker et al 1967 (8) showed that an acute ammonia load in normal rats resulted in significant decreases of ATP and phosphocreatine contents in the brainstem. However, in rats subjected to a porta-caval shunt, thereby becoming moderately hyperammonemic, the energy charge of the brain tissue was unchanged

5 weeks after surgery (9). In studies performed 3 hours after hepatectomy the energy state of the brain was also preserved (10). In rats with a porta-caval shunt subjected to an acute ammonia load, the ATP/ADP ratio was unchanged in precomatose but significantly lower in comatose rats (11).

Ammonia is detoxified in the brain by the reductive amination of α -ketoglutarate to glutamate and the amidation of glutamate to glutamine (Fig 1). Thus, detoxification of ammonia requires α -ketoglutarate which theoretically could affect the citric acid cycle resulting in impaired energy production (12). In rats subjected to hepatectomy (10) or ammonia intoxication (13), the α -ketoglutarate levels have been shown to be unchanged or even increased (14) while rats with a porta-caval anastomosis have significantly lower levels of α -ketoglutarate in the brainstem 5 weeks after surgery (9).

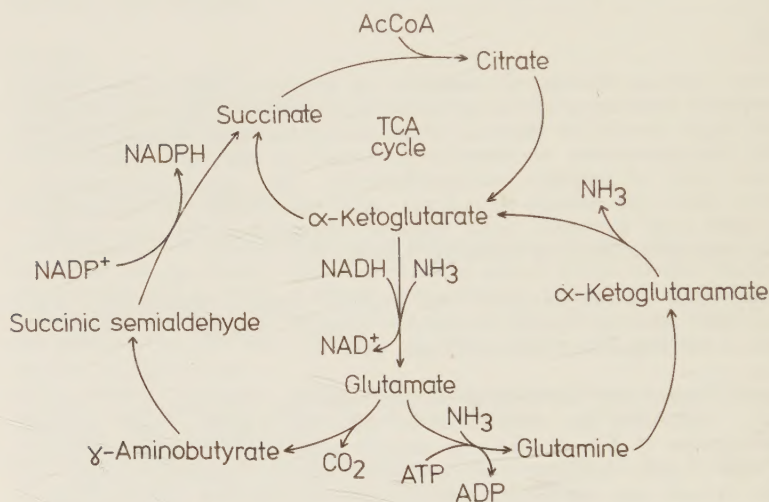


Fig 1. Schematic representation of the citric acid cycle and the detoxification of ammonia in the brain.

Glutamic acid has also been suggested to play a role in the pathogenesis of hepatic encephalopathy, since it is a putative excitatory neurotransmitter (15). Decreased glutamate concentrations in the brain have been demonstrated after porta-caval shunt with an acute ammonia load (11) and hepatectomy (10). However, hepatectomized rats with body temperature above 33°C had unchanged levels of glutamate and rats below 33°C had lower levels 6 hours after surgery (16). Rats with a porta-caval shunt have been shown to have

low (9), unchanged (9, 17) or even high (18) levels of glutamate in the brain. Hepatic devascularization in the rat results in unchanged levels of glutamate in the brain (19).

Increased concentrations of glutamine in the brain are consistently seen in experimental animals after portacaval shunt (17, 18), hepatectomy (16, 20), porta-caval shunt with an acute ammonia load (11, 21), ammonia intoxication in normal rats (21), and hepatic devascularization (19). Tyce et al (20), however, have recently shown that the synthesis of glutamine in the brain is inhibited after hepatectomy and suggested that the increased concentration is caused by an even greater inhibition of degradation rather than increased synthesis. In patients, elevated levels of glutamine in CSF correlate with the grade of encephalopathy (22). Glutamine is taken up by nerve endings and may have some central depressant effect by itself or act by replacing other neurotransmitters (23). Increased CSF levels of α -ketoglutarate, the deaminated metabolite of glutamine have also been demonstrated in hepatic encephalopathy (24).

Gamma-aminobutyric acid (GABA) which is synthesized by decarboxylation of glutamate is an inhibitory neurotransmitter and has been proposed to play a role in encephalopathy (25, 26). Rats with a porta-caval shunt loaded acutely with ammonia or rats with chemically induced liver damage have unchanged levels of GABA in the brain (11,25). Mans et al (19) showed increased GABA levels after hepatic devascularization in the rat. Recently, Schafer et al (26) demonstrated high levels of GABA in plasma and an increased number of GABA receptors in the brains of rabbits with galactosamine-induced hepatic encephalopathy. The suggested increase of GABA levels in the brain was considered to originate in the colon and penetrate a permeable blood-brain barrier.

Hyperammonemia in various experimental models is also associated with decreased levels of aspartate in the brain (9, 11, 19, 20). The reason for the lower levels of aspartate is not clear. Hindfeldt et al (11) found that the decreased aspartate levels were associated with increases of alanine and malate and a decrease of oxalacetate when they studied rats with a porta-caval shunt subjected to an acute ammonia load. The authors suggested that these changes may result from a concerted transamination mechanism facilitated by increased pyruvate.

Thus, the toxic effects of ammonia may be explained by the detoxification reactions. However, different experimental and analytical conditions, the presence of regional variations in brain metabolism, and missing information about physiological variables make the interpretations difficult.

Short-chain fatty acids

Fatty acid metabolism is deranged in patients with liver disease

and short-chain fatty acids are increased in blood and breath of patients with hepatic encephalopathy (27-29). Intravenous injection of valeric and isovaleric acids produced coma in rabbits (30). However, the correlation between short-chain fatty acid levels and the grade of encephalopathy is not good (31). In addition, administration of short-chain fatty acids in cirrhotic patients failed to provoke encephalopathy (32).

Mercaptans

Patients with liver failure and hepatic encephalopathy have increased concentrations in blood of methanethiol (methyl mercaptan) (33). Methanethiol is formed from methionine and metabolized in the liver. In portosystemic shunting methanethiol bypasses the liver and is excreted in urine and breath. Blood concentrations of methanethiol have been reported to correlate well with the clinical grade of encephalopathy (33).

The synergistic hypothesis

Coma induced by ammonia and methanethiol individually in normal animals required doses resulting in higher concentrations of these compounds than did surgically produced hepatic coma (34,35). Therefore it was suggested that smaller amounts of ammonia, methanethiol and short-chain fatty acids administered together would provoke hepatic encephalopathy by acting synergistically (34, 35). Thus normal rats became comatose when injected with a suitable mixture of ammonia, methanethiol and octanoic acid resulting in lower brain ammonia and methanethiol concentrations than seen in rats with surgically induced hepatic coma (35, 36). However, the mechanisms of the neurotoxic actions of these substances have not been elucidated.

The amino acid neurotransmitter hypothesis

Bollman et al 1924 (37) showed that formation of urea ceased immediately after the removal of the liver in the dog and concluded that the liver was directly concerned with the deamination of amino acids. In subsequent studies of anhepatic dogs an increase in the amino acid nitrogen content of the blood (38) and increases in amino acids by chromatographic technique (39) were demonstrated. Several authors have later reported disturbed metabolism of plasma and brain amino acids in hepatic failure of experimental animals and humans. Total hepatectomy in the dog results in increased levels of most plasma amino acids with the exception of the branched chain amino acids (BCAA) valine, leucine and isoleucine which are below normal values (40). The high levels of some amino acids probably reflect protein break-down in peripheral tissues and absence of liver function. The low plasma levels of BCAA after hepatectomy may partly be caused by extrahepatic metabolism of these amino acids (41).

In liver ischemia, almost all amino acids including the BCAA were elevated (19). Mazziotti et al (42) compared liver devascularization with hepatectomy in pigs. The aromatic amino acids, methionine and tryptophan increased immediately after liver ischemia. On the other hand, the levels of aromatic amino acids were not increased until 18 hours after hepatectomy. Animals with liver ischemia also went into coma earlier than hepatectomized animals. It was concluded that necrotic hepatocytes were the major source of elevated plasma amino acids after ischemia. The onset of coma was related to increases in the aromatic amino acid levels rather than to increased blood ammonia values.

Patients with acute hepatic failure have high concentrations of several amino acids in plasma (43-46). The BCAA levels remain normal in acute hepatic necrosis with a previously normal liver and are decreased in chronic liver disease with superimposed acute insults (46).

In chronic liver disease in man or in rats with portosystemic shunting, the concentrations of phenylalanine, tyrosine, free tryptophan but not necessarily total tryptophan, methionine and histidine are elevated and the BCAA concentrations low (47, 48). This amino acid imbalance has partially been related to a catabolic state with hyperglucagonemia and hyperinsulinemia (49-51). Elevated levels of tyrosine may be related to endogenous protein break-down and reduced hepatic oxidation capacity (52). Fischer et al (53) have proposed the ratio

$$\frac{\text{valine} + \text{leucine} + \text{isoleucine}}{\text{phenylalanine} + \text{tyrosine}}$$

to correlate with the grade of encephalopathy. This ratio is normally 3.0 to 3.5 and in portosystemic encephalopathy below 1.0. However, subsequent studies have not confirmed the suggested relationship between the ratio and the grade of encephalopathy (54).

The BCAA valine, leucine and isoleucine have been shown to compete with other large neutral amino acids (LNAA) across the blood-brain barrier at a common transport system (55-58). Thus, lower levels of BCAA and high levels of aromatic amino acids may facilitate entry of aromatic amino acids into the brain.

High concentrations of several amino acids including phenylalanine, tyrosine and tryptophan in brain tissue and CSF of humans with hepatic failure (45, 59) and in the brain of animals after hepatectomy (16) or hepatic devascularization (19) have been observed. According to Record et al (45) brain to plasma ratios of most amino acids in patients with hepatic coma were similar to the corresponding ratios in control subjects, and it was suggested that plasma concentrations was the main factor controlling the cerebral concentration. This is in contrast to findings in rats with a portacaval shunt where the increased brain tissue levels of tyrosine,

phenylalanine and tryptophan tissue could not be explained only by plasma levels or altered competition (47). In these animals an increased transport of neutral amino acids from plasma to brain was shown. The reason for this increased transport activity is unknown. Recently, it has been suggested that high concentrations of glutamine in the brain of these rats may stimulate the transport of other neutral amino acids across the blood-brain barrier. This suggestion is supported by the excellent correlation between high levels of glutamine and these amino acids in the brain (60). These data have resulted in the so-called "unified hypothesis" concerning the etiology of hepatic encephalopathy, since glutamine is probably the main product of ammonia detoxification (60).

In 1971, Fischer and Baldessarini (61) proposed "that some of the neurological and cardiological manifestations of hepatic failure may be due to the accumulation of false neurochemical transmitters replacing normal transmitters in the peripheral and central nervous system". Thus, in portosystemic shunting and hepatic insufficiency, aromatic amino acids and their amines bypass the liver and accumulate in the blood and the central nervous system. It was later considered that these amines could also be synthesized in the brain (62) and that some of them do not penetrate the blood-brain barrier in appreciable amounts (55). However, the catecholamine precursors phenylalanine and tyrosine may penetrate the blood-brain barrier. Increased concentrations of the "false neurotransmitters" octopamine and phenylethanolamine and decreased concentrations of dopamine and norepinephrine have been suggested to result from an excess of aromatic amino acids in the brain (63) (Fig 2). High concentrations of phenylalanine in the brain may inhibit the synthesis of dopa from tyrosine by competing with tyrosine for the enzyme tyrosine hydroxylase (63, 64). Thus, the high levels of phenylalanine and tyrosine may result in increased phenylethylamine and tyramine concentrations by decarboxylation. Subsequently, these amines are β -hydroxylated to form the potential false neurotransmitters, β -phenylethanolamine and octopamine which may displace norepinephrine from neuronal storage sites. Phenylethylamine and tyramine may also competitively inhibit the β -hydroxylation of dopamine to norepinephrine (63).

Several clinical and experimental studies have given support to this theory. Accumulation of octopamine in brain and blood of rats with liver ischemia and porta-caval shunt (62, 65) has been demonstrated. In patients with portosystemic shunting and encephalopathy, increased levels of octopamine in serum and urine have been shown to correlate with the grade of encephalopathy (66). Infusion of octopamine intraventricularly in the rat raised octopamine concentrations more than 20,000 fold and resulted in 90% decreases of brain norepinephrine and dopamine levels without inducing coma (67). However, the significance of these results is difficult to evaluate because of the unphysiologic amounts of octopamine used.

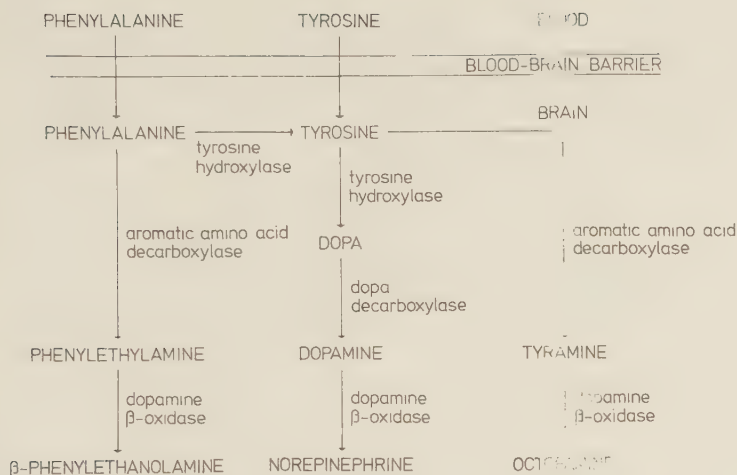


Fig 2. Catecholamine synthesis in the brain.

Tyramine, the precursor of octopamine is elevated in the brains and CSF of dogs with a porta-caval shunt and the levels are significantly higher in dogs with stage II and III encephalopathy than in dogs with stage 0 and I encephalopathy (68).

Phenylethanolamine has been shown to be increased in the cisternal CSF of dogs with a porta-caval shunt and encephalopathy (69) as well as in plasma of humans with liver failure (70).

The dopamine levels in brain tissue have been shown to be near-normal in hepatectomized rats (71) and low in dogs with a porta-caval anastomosis (68) or in humans with hepatic coma (72). In CSF of dogs with a porta-caval anastomosis the levels of dopamine are high (68), and in patients with fulminant hepatic failure homovanillic acid, a metabolite of dopamine, was increased (73).

Decreased levels of norepinephrine in the brain have been demonstrated in rats with a devascularized liver (68), after hepatectomy (71) and in dogs with a porta-caval shunt and encephalopathy (68). Tyce and Owen (71) observed low norepinephrine levels and near-normal dopamine levels in the brain of hepatectomized rats. They suggested an inhibited norepinephrine synthesis due to inhibition either of dopamine uptake by vesicles or of the hydroxylation of dopamine. Dopamine and norepinephrine have been observed to have both excitatory and inhibitory effects. Apparently, it is not clear whether or not an ascending noradrenergic system participates in the mechanism of arousal or the maintenance of wakefulness as discussed by Krnjevic 1974 (74).

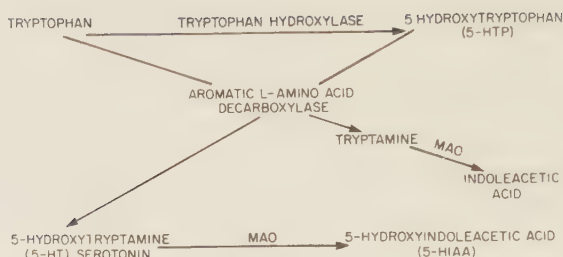


Fig 3. Serotonin synthesis in the brain.

High concentrations of tryptophan in the brain tissue may increase the synthesis and turnover of serotonin in patients with hepatic failure (45, 75) and in pigs (76), dogs and rats (19, 77, 78) after hepatic devascularization or complete hepatectomy (Fig 3). The transport of tryptophan into the brain may depend on the concentration of free tryptophan (unbound to albumin) and on competition with other neutral amino acids (59, 79). A decrease of serum albumin and an increase of non-esterified fatty acids displacing tryptophan from albumin, may contribute to the elevated levels of free tryptophan (59, 80). However, the mechanism for the transport of tryptophan across the blood-brain barrier has not been fully clarified. Mans et al (81) suggested that the tryptophan transport may increase via a nonsaturable route. In patients with hepatic coma, high concentrations of serotonin and 5-hydroxyindoleacetic acid (5-HIAA) have been reported to exist in the reticular formation and raphe nuclei of the brain stem (75), and high concentrations of tryptophan and 5-HIAA have been observed in the CSF (59, 73, 82). In pigs with liver devascularization, brain concentrations of tryptophan and 5-HIAA were high and serotonin unchanged 5½-9 hours after surgery (76). In the brains of rats 12-24 hours after hepatectomy, high concentrations of tryptophan, serotonin and 5-HIAA were demonstrated (77). In the study of Ono et al (59) the CSF levels of tryptophan but not phenylalanine and tyrosine were higher in patients with coma than in non-comatose cirrhotic patients. However, in later studies it was shown that tryptophan and 5-HIAA concentrations did not consistently change with recovery in patients with hepatic encephalopathy (82). Serotonin has been implicated in the regulation of consciousness in the brain stem (83). This substance may also compete with norepinephrine for storage in adrenergic neurons (84). Tryptophan may inhibit the enzymatic hydroxylation of tyrosine to dopa and 5-hydroxytryptophan could compete with dopa for decarboxylation by dopa decarboxylase (63). However, infusion of tryptophan in the carotid artery could not

provoke coma in dogs (85). Reproducible coma was obtained only if phenylalanine was added to tryptophan.

The amino acid neurotransmitter hypothesis of hepatic encephalopathy has lead to therapeutic attempts to correct the plasma amino acid imbalance and the suggested derangement in neurotransmitter levels.

Branched chain amino acids (BCAA)

Fischer et al 1975 (86) attempted to correct the deranged amino acid pattern in hepatic encephalopathy by infusing an amino acid solution enriched with BCAA (F080) in dogs with a porta-caval anastomosis and encephalopathy. Infusion of the solution improved encephalopathy and at the same time plasma aromatic amino acid levels decreased. On the other hand, infusion of a standardized synthetic amino acid solution (Freamine II) resulted in the death of most of the dogs.

In subsequent studies by Smith et al 1978 (69), parenteral administration of BCAA enriched amino acid solution to dogs with porta-caval anastomosis and encephalopathy improved encephalopathy and normalized amino acid concentrations in plasma and CSF. In addition, the CSF concentrations of octopamine and phenylethanolamine decreased. Freund et al 1980 (85) demonstrated that simultaneous infusions of phenylalanine and tryptophan together with BCAA in the carotid artery could prevent coma.

Infusion of a BCAA enriched amino acid solution (F080) together with 23% dextrose in patients with chronic liver disease and superimposed insult resulted in improvement of the encephalopathy and normalization of the plasma amino acid levels. In patients with fulminant hepatitis and a previously normal liver, improvement of the encephalopathy was observed only in one of three patients (53).

Riederer et al (75) reported four patients in hepatic coma who regained consciousness 1-3 hours after receiving a solution containing 5% valine. These patients later died of gastrointestinal bleeding or cardiac failure. In the raphe nuclei and reticular formation in these patients, significantly lower concentrations of tryptophan, serotonin, 5-HIAA, and ammonia were observed than in patients receiving ordinary parenteral nutrition. In the study of Rössle et al (87), patients with hepatic encephalopathy stage II-III, infusion of the BCAA enriched amino acid solution resulted in improvement of encephalopathy and a rapid fall of aromatic amino acids in plasma and of ammonia and aromatic amino acids in CSF. Several other authors have reported promising results with BCAA infusion in hepatic encephalopathy (88-92). However, in two randomized studies no favourable effect was demonstrated (93, 94). In the randomized multicenter study of Wahren et al (94), BCAA did not significantly improve cerebral function and significantly more patients died in the BCAA treated group.

Oral administration of BCAA improved encephalopathy and nitrogen balance according to several studies (95-98). The study of Horst et al (98) was a double-blind randomized comparison of dietary protein and an oral BCAA supplement. In a double-blind cross-over study of Eriksson et al (99), however, no improvement was reported.

The decreased levels of some amino acids in plasma and of amino acids and amines in CSF after administration of BCAA are not explained but are probably the result of both peripheral and central mechanisms. Thus, BCAA compete with other large neutral amino acids at a common transport system (55-58) at the blood-brain barrier. Peripheral effects may be related to an influence on protein metabolism in muscular tissue. In vitro experiments have demonstrated that leucine stimulates protein synthesis and inhibits protein breakdown in incubated skeletal muscle (100, 101). In the posttraumatic rat, BCAA may stimulate protein synthesis both in muscle and in liver (102). In vivo studies in humans, however, have been controversial. According to Sherwin (103), intravenous administration of leucine in prolonged fasting results in improvement in nitrogen balance and unchanged excretion of 3-methylhistidine. In another study (104) perorally administered leucine increased glutamine and total nitrogen release from forearm muscle in healthy subjects.

L-dopa and bromocriptine

Treatment with L-dopa and bromocriptine aims at restoring derangement of certain brain neurotransmitters. L-dopa, the precursor of dopamine improves encephalopathy according to several authors (105, 106). One prospective randomized study failed, however, to confirm any effect compared to placebo (107). It has also been shown by Zieve et al (108) that intragastric administration of L-dopa to rats could prevent coma induced by intra-peritoneal administration of ammonia. They attributed this to a peripheral effect with increased renal excretion of ammonia and urea. Bromocriptine, a dopamine receptor agonist, has also been used in attempts to improve chronic portosystemic encephalopathy but the results are inconsistent (109, 110).

EXPERIMENTAL MODELS

Experimental models of liver failure and encephalopathy include chemically induced liver damage, porta-caval shunt, hepatic devascularization and hepatectomy. In order to surgically induce coma in the rat hepatic devascularization or hepatectomy are necessary. These procedures give rise to several metabolic disturbances, of which some are similar to those seen in man with acute hepatic failure.

Several techniques for hepatectomy in experimental animals have been described. A three stage procedure for total hepatectomy in the dog was described by Mann in 1921 (111). This technique involves side-to-side porta-caval shunt and ligation of the inferior vena cava in the first stage, ligation of the portal vein in the second stage and complete removal of the liver in the third stage. In 1927 Markowitz and Soskin (112) described a two stage technique with partial ligation of the inferior vena cava and the portal vein in the first stage and total hepatectomy in the second. Meehan (113) adapted this procedure for the rat in 1954. By using cellophane for the partial ligatures a progressive constriction of the portal and caval veins was obtained (114).

In 1968, Bollman and van Hook (115) employed cellophane banding of the portal vein in the rat and ligated the inferior vena cava in the first stage followed by total hepatectomy in the second. Survival at 8 hours after hepatectomy was 90% and at 24 hours 60% with body temperatures of about 30° and 27°C, respectively. The survival time was markedly reduced if the body temperature was upheld.

Bismuth et al 1968 (116) presented a three stage procedure implying ligation of the inferior vena cava in the first stage, construction of a porta-caval shunt 3 months after the first stage and total hepatectomy after an additional 48 hours. This procedure has also been used by Degos 1970 (117) and Franco and Bismuth (118) who achieved a mean survival time of 23 hours in rats with a body temperature maintained at 28°C.

Most of these different techniques for total hepatectomy have been used in metabolic studies. Hepatic devascularization in two stages has also been used (42, 62, 78). However, hepatectomy generally results in a longer survival time (42, 118). Both hepatectomy and hepatic devascularization imply a surgical trauma and result in metabolic and physiologic changes. However, few methods remain if metabolic changes after acute liver failure are to be studied experimentally, particularly if potential side effects of pharmacological agents are to be avoided.

AIMS OF THE PRESENT STUDY

The pathogenetic mechanisms in hepatic encephalopathy are still not clear, although portosystemic shunting of blood and disturbed ammonia and amino acid metabolism have been proposed to play important roles. Accepted treatment of hepatic encephalopathy includes attempts aimed at lowering ammonia levels in the blood and, more recently, administration of BCAA which might partially correct plasma amino acid imbalance. However, little is known about the effects on amino acid levels and neurotransmitter concentrations in the brain after administration of BCAA to animals with experimentally induced acute hepatic failure.

Acute hepatic failure and encephalopathy may be studied in surgically created experimental models such as hepatic devascularization or total hepatectomy. In the present study, total hepatectomy was chosen for several reasons. Hepatectomy generally results in a longer survival time (42, 118). In models, in which the liver is devascularized and not removed, the necrotic tissue may release large quantities of amino acids, thus masking the release of amino acids into the circulation from the periphery. Therefore, apart from being a valuable model for metabolic studies of the brain tissue in fulminant hepatic failure, hepatectomy provides a model in which the effects of infusing branched-chain amino acids on peripheral metabolism can be studied.

The purpose of the present work was

- (I) to develop a safe and reliable surgical technique for complete removal of the liver in the rat
- (II) to determine simultaneously the early effects of total hepatectomy on cerebral energy state, ammonia-related amino acids in the brain and plasma amino acids
- (III) to study the effects of administration of BCAA either individually or in combination on brain and plasma amino acid levels and on indoleamine levels in brain tissue of hepatectomized rats
- (IV) to study plasma and brain amino acid and brain catecholamine concentrations after administration of glucose alone or in combination with BCAA 6 and 18 hours after hepatectomy
- (V) to study norepinephrine and dopamine levels in eight different brain regions after administration of glucose alone or in combination with BCAA 18 hours after hepatectomy

MATERIAL AND METHODS

Animals

Male Wistar rats (Møllegaard's Breeding Center, Ejby, Lille Skensvid, Denmark) with an initial body weight of 180-225 g were used in paper II while male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA) weighing 200-275 g were used in paper III-V. The animals were housed 2 per cage and kept in a colony room at $21 \pm 1^{\circ}$ under 12 hr light-dark cycle. Tap water, rat chow (Astra Ewos, Södertälje, Sweden) in paper II and Purina rat chow (Ralston Purina Co., St. Louis, Mo, USA) in paper III-V were available ad libitum. Following the last surgical procedure the rats had access only to tap water.

Surgical procedures

The animals underwent hepatectomy in three stages and all surgical interventions were carried out under ether anesthesia (Fig 4).

First stage operation. The abdomen was opened through a midline incision. The inferior vena cava was exposed and freed from surrounding structures. The vein was doubly ligated with 4-0 Dexon[®] just above the right renal vein and divided between the ligatures. The abdominal incision was closed in two layers with interrupted 4-0 Dexon[®] sutures.

Second stage operation. Four weeks after the first stage, a conventional or modified end-to-side porta-caval anastomosis was constructed. With the aid of an operating microscope (Zeiss OpMi-1 or 6), it was possible to choose the proper location for the shunt below the thrombotic changes that consistently developed close to the lower ligature of the inferior vena cava. Because of these changes microsurgical technique was essential to obtain a successful result of the shunting procedure. Conventional porta-caval shunting was used in paper II. The shunt was constructed with continuous 9-0 Ethilon[®] sutures and 10X magnification. In all other respects, the technique described by Holmin and Siesjö (9) was used. This technique implies incision in the anterior-medial part of the inferior vena cava and clamping of the superior mesenteric artery during performance of the shunt to diminish intestinal congestion. In paper III-V the same technique was used with one exception. Instead of ligating doubly and dividing the gastroduodenal vein (pancreatico-duodenal vein), the flow through this vein was preserved.

Third stage operation. One week after the second stage, a total hepatectomy was performed. The procedure implied ligatures and division of the hepatic artery, the biliary duct, and finally, the inferior vena cava close to the diaphragm. Also in this stage microsurgical technique and operating microscope or magnifying glasses were used to minimize trauma and blood loss.

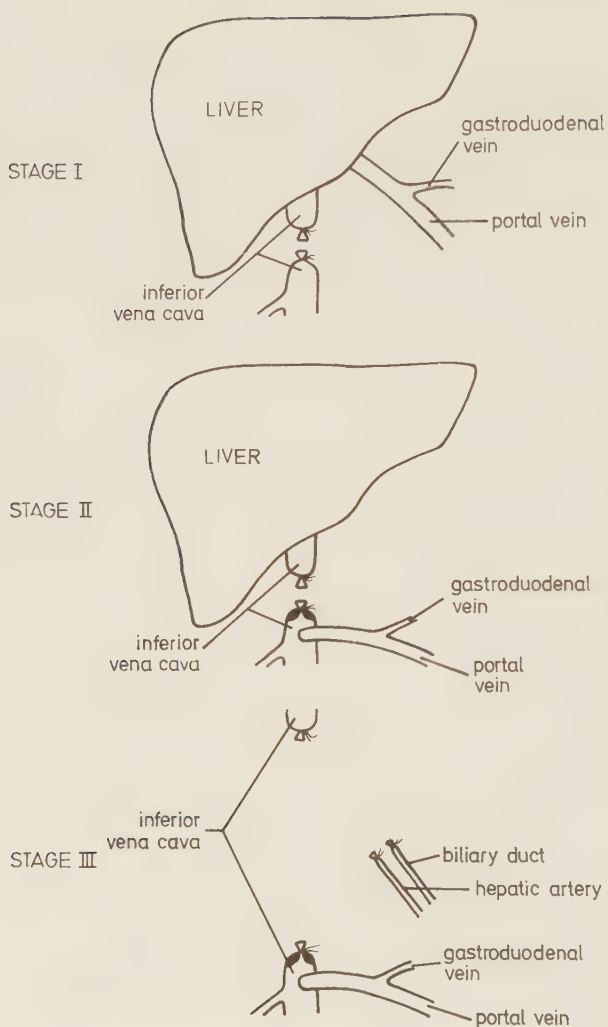


Fig 4. Total hepatectomy in three stages. Stage I: Double ligature and division of the inferior vena cava. Stage II: Porta-caval shunt 4 weeks later. Stage III: Total hepatectomy one week after stage II.

Two control groups were used:

Control a: These rats underwent stage I and II of the hepatectomy. Stage III was replaced by the sham operation that involved all steps of the hepatectomy procedure with the exception of the ligatures. The sham operation had the same duration as stage III.

Control b: Stage I was performed as above. Stage II and III were replaced by comparable sham operations.

Experimental procedures

The rats were housed in individual cages after the third surgical procedure. Body temperature was kept close to 29°C in hepatectomized and control animals (II).

In the remaining animals (III-V) the body temperature was kept at 35-37°C by a heating lamp.

Paper II: Glucose in 0.9% saline was given i.p. after the hepatectomy every half hour until the end of the experiment (60-80 mg glucose per 100 g body weight and hour). Three and a half hours after hepatectomy or sham operation (control a) the rats were re-anesthetized with ether, tracheotomized and maintained on 70% nitrous oxide and 30% oxygen. The animals were immobilized with i.p. tubocurarine chloride and ventilated with a Starling type respirator which was set to give arterial CO₂ tensions of about 29 mmHg. The right femoral artery was cannulated for blood pressure measurements and for anaerobic sampling of blood. The animals were heparinized. The atlanto-occipital membrane was exposed and a plastic funnel fitted into a skin incision over the skull bone for subsequent freezing of the brain *in situ*. Four hours after the hepatectomy or the corresponding sham operation the tissue was frozen by pouring liquid nitrogen into the funnel (119). When the brain was frozen, it was chiselled out, using cold instruments, and stored at -85°C until analysed.

Paper III-V. At the last operation, the rats were provided with a silastic catheter in the left jugular vein. Various solutions were infused by using an infusion pump. The rats were infused with 20% or 10% glucose solutions to prevent hypoglycemia. The solutions contained electrolytes as follows: 145 mmol/l NaCl, 13 mmol/l K₂HPO₄, 2.3 mmol/l calcium gluconate, 2 mmol/l MgSO₄ and 1.5 mmol/l ZnCl₂.

The rats were killed by decapitation at 6 hours (III, IV) and at 18 hours (IV, V) after hepatectomy or sham operation. Blood from the cervical wound was collected in heparinized beakers. Plasma was separated by centrifugation and kept frozen at -72°C. The brain was removed, divided into two hemispheres and immediately frozen on dry

ice (III, IV). In paper V the brain was removed and dissected into the following regions: cortex (2 hemispheres), hypothalamus, striatum, hippocampus, mesencephalon, remaining diencephalon, pons-medulla, and cerebellum. The dissection was carried out on a chilled glass plate and the brain samples were immediately frozen in liquid nitrogen. The regional brain dissections were performed according to Glowinski and Iversen (120) with the following exceptions:

1. Mesencephalon was separated from the remaining part of diencephalon and the septal area by a vertical cut just anterior to the superior colliculus.
2. The hypothalamic area was dissected to a depth of approximately 2.5 mm from the optic chiasm to the posterior mamillary area and bounded laterally by the choroid fissure.
3. Cortex from one cerebral hemisphere was used for catecholamine analyses and the other hemisphere for amino acid analyses.

Paper III. Eight groups (A-H) of rats were used. Group A-F underwent hepatectomy, group G and F were control a and b, respectively. Group A, G, and H received a solution containing 20% glucose and electrolytes as above. The remaining groups received the same solution to which BCAA were added as follows: Group B 0.1 mol/l BCAA (0.033 mol/l of each valine, leucine and isoleucine; Group C 0.1 mol/l valine; Group D 0.1 mol/l leucine; Group E 0.1 mol/l isoleucine; and Group F 0.3 mol/l BCAA (0.1 mol/l of each valine, leucine and isoleucine). The solutions were infused at a rate of 0.6 ml/hr/100 g body weight.

Paper IV. Four groups (A-D) of rats were used. Group A and B were hepatectomized and Group C and D were controls. Group C was equal to control a and Group D to control b.

The hepatectomized animals were infused either with a solution containing 20% glucose and electrolytes as above (Group A) or the same solution plus 0.24 mol/l BCAA (0.08 mol/l of each valine, leucine and isoleucine, Group B). The control groups C and D received the glucose solution. All solutions were infused at a rate of 0.6 ml/hr/100 g body weight.

Paper V. Four groups (I-IV) were used. Group I-II were hepatectomized while group III (control a) and IV (control b) were controls. Postoperatively the rats in Groups I, III and IV received a solution containing 10% glucose and electrolytes. Group II received the same solution with 0.24 mol/l BCAA added (0.08 mol/l of each valine, leucine and isoleucine). The solutions were infused at a rate of 0.9 ml/hr/100 g body weight.

Analytical techniques

PO₂, PCO₂ and pH (paper II). PO₂, PCO₂ and pH in arterial blood were measured every 10 minutes after tracheostomy of the rats. The samples were analysed immediately using microelectrodes operated at 30°C (Radiometer, Copenhagen and Eschweiler & Co., Kiel) with appropriate temperature corrections.

Brain metabolites in paper II. Brain samples were stored at -85°C until analysed. The brains were dissected and weighed at -20°C. Cerebral cortical tissue from the frontoparietal region and the brain stem were extracted at this temperature with HCl-methanol and subsequently with perchloric acid at 0°C. The extracts were neutralized with KOH-imidazole mixture as previously described (121).

ATP, ADP, AMP, phosphocreatine, creatine, glutamate, glutamine, γ -aminobutyric acid (GABA), aspartate, alanine, glycogen, glucose, lactate, pyruvate, α -ketoglutarate and malate were determined with fluorometric techniques (122). Analytical conditions have been previously described (123-126).

Amino acid analyses. In paper II, plasma was deproteinized by mixing with an equal volume of 7.5% sulfosalicylic acid (SSA) (pH 1.7) containing an internal standard (norleucine). After vortex mixture, the samples were centrifuged at 30 000X g at 4°C for 20 minutes. The supernatant was passed through a 0.45 micron filter (Millipore) and analysed directly. Quantitative determinations of amino acids were carried out by a column chromatographic technique with a JLC-6AH (JEOL) automatic amino acid analyser, using a lithium buffer system.

In paper III-V plasma and brain amino acids were determined by a Beckman 121-MB amino acid analyser with automatic integration. Plasma was deproteinized by mixing 0.5 ml plasma with 1.5 ml 5% SSA, pH 1.7, containing 133 nmol/ml thienylalanine as an internal standard. After vortexing, the samples were centrifuged at 30 000X g at 4°C and passed through a 0.45 micron Millipore filter. The brain was homogenized in 3 times its weight of 5% SSA (pH 1.4), containing 133 nmol/ml thienylalanine. Each brain sample was analysed twice, once at the 1:4 dilution of the homogenate for the low-concentration amino acids and, again, after a second 1:25 dilution to measure the high-concentration amino acids glutamate, glutamine, and aspartate.

Indoleamines in brain (paper III). Serotonin and 5-HIAA in the brain were determined fluorometrically after extraction of the indoles in butanol and heptane (127). Brain tryptophan was assayed by a revised fluorometric method (128).

Catecholamines (paper IV and V). Norepinephrine and dopamine were assayed by liquid chromatography with electrochemical detection from one half of the brain (IV) or from each region (V) as follows: brain tissue was homogenized in 5 volumes of 0.4 mol/l HClO_4 containing 5 mmol/l sodium metabisulphite, 1.5 mmol/l EDTA and 100 ng/ml of alpha-methyl norepinephrine as an internal standard. After centrifugation (20 000X g, 20 min, 4°C) catecholamines were absorbed into 100 μg of activated alumina and the pH was adjusted to 8.1-8.4 with 0.5 mol/l Tris buffer, pH 10. The alumina was washed twice with distilled water and the catecholamines were eluted with 1 ml of 0.1 mol/l HCl. Twenty-five μl were injected onto the column. The mobile phase buffer was composed as follows: 10% MeOH, 90% 0.1 mol/l KH_2PO_4 , 0.2 mmol/l sodium octanesulphonic acid, 0.1 mmol/l EDTA, pH 3.0, delivered at a flow rate of 1.1 ml/min.

Glucose. Plasma glucose was assayed using a kinetic glucose-oxidase method of JL 919 at 37°C (Instrumentation Labs, Inc.).

Statistics

The data in paper II were subjected to Wilcoxon's rank sum test. The data in paper III and V were evaluated with analysis of variance performed for all groups together and also separately for the glucose treated groups without BCAA. Follow-up tests were performed with Newman-Keul's test. In paper IV a multivariate analysis of variance (MANOVA) for plasma or for brain amino acids and catecholamines in brain was performed for the following factors: treatment, time, treatment x time. Treatment includes surgery and infusion. The following amino acids were studied: aspartic acid, threonine, serine, glutamic acid, glutamine, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, lysine, histidine and arginine. Overall significance in this MANOVA was precedent for univariate 4×2 factorial analysis of variance (ANOVA) for each amino acid and catecholamine in plasma or brain. Comparisons between the groups utilized the Newman-Keul multiple range test. For plasma glucose and tryptophan, analysis of variance was carried out separately.

Chi square test and Student's t-test were used when appropriate (IV and V).

RESULTS AND DISCUSSION

A microsurgical method for total hepatectomy in the rat (I)

The presented technique is similar to that described by Bismuth et al (116) but differs from the French model in some important respects. Instead of using a single ligature of the inferior vena cava in the first stage, the vessel was divided between two ligatures thereby facilitating the hepatectomy in the third stage. In all rats intended for 6 and 18 hours of experiments the end-to-side porta-caval shunt was carried out in a modified way; i.e. the gastroduodenal (pancreatico-duodenal) vein was not ligated. In the light of recently published data showing the importance of the blood in the gastroduodenal vein (129) it is possible that this modification of the conventional porta-caval shunt makes the rats more capable of tolerating the third stage of hepatectomy. Finally, the use of microsurgical techniques in both the second and third stage is probably of great benefit, as they are atraumatic and minimize blood loss.

90% of the rats survived the first stage of the hepatectomy. The reason for the mortality is unclear. It has previously been suggested that rats succumbing after ligation of suprarenal vena cava die early in hyperkalemia or later in a uremia (130). The rats gained weight and behaved normally.

Construction of the porta-caval shunt was generally easy to perform. In some cases extensive thrombotic changes in the inferior vena cava could extend the clamping time of the portal vein. A clamping time less than or equal to 20 minutes resulted in survival in all cases.

A total of 165 rats has been subjected to hepatectomy. Twenty-five of 30 rats have been studied after 4 hours. Some of these rats died early after the hepatectomy during the development of the technique. All 61 rats survived to the 6 hour studies and 65% (48 of 74) survived to the 18 hours' studies. The rats studied at 4 hours were supported by glucose i.p. and the rats studied at 6 and 18 hours were supported with i.v. administered glucose solution to prevent hypoglycemia. Addition of various amino acids to the glucose solution did not significantly alter the survival rate at 18 hours. Apart from those rats studied at 4 hours, all rats were kept warm (35-37°) by using a heating lamp. Thus, the present technique enables studies of "warm" (35-37° C) rats at 18 hours with a relatively low mortality rate. With previously described techniques for total hepatectomy in rats kept at a near-normal temperature, such high survival rates have not been reported at 18 hours after the operative procedure.

Hepatectomy results in a declining body temperature and it has been shown that when environmental temperature is 20° C, the body temperature of the rat declines to about 30° C in 6 hours and declines

about 1° C more for every 6 hour period (115). It is also evident that the survival rate is considerably lower if body temperature is upheld (115). Administration of glucose is very important and Meehan (113) could increase the survival rate by increasing the amounts of glucose administered.

Cerebral energy state, ammonia related amino acids of the brain and plasma amino acids 4 hours after hepatectomy (II)

The ammonia metabolism is deranged in hepatic encephalopathy. Previous experimental studies have suggested that ammonia interferes with the brain energy state. However, conflicting data exist as to whether hyperammonemia leads to an unbalanced energy state of the brain tissue or not (8-11). In brain tissue or CSF, glutamine levels are consistently elevated (11, 17, 19, 20, 21, 22) and aspartate levels decreased (11, 19, 20), whereas glutamate levels have been reported to be decreased (9, 10, 11, 16), unchanged (9, 16, 17) or elevated (18) in different experimental models under different experimental conditions. Plasma amino acid imbalance and a rise in brain/plasma concentration ratios have also been suggested to play a role in the pathogenesis of hepatic encephalopathy (47, 60).

All the animals in the present study were awake 3½ hours after hepatectomy before reanesthesia. At the end of the experiment arterial PO₂, PCO₂, pH and body temperature did not significantly differ between the groups. Hepatectomized rats had a significantly lower mean arterial blood pressure than the shunted controls.

In the cerebral cortex of hepatectomized rats the concentration of lactate and the lactate/pyruvate ratio were significantly increased. The glycogen concentration was decreased. The concentrations of glucose, pyruvate, α-ketoglutarate and malate were unchanged when compared with the corresponding values in the shunted controls. In the brain stem there was no significant difference between the groups in any of these metabolites. The concentration of phosphocreatine, creatine, ATP, ADP and AMP in the cerebral cortex and brain stem did not differ significantly between the groups with the exception of a slightly elevated ATP concentration in the anhepatic group.

The concentrations of glutamate and aspartate were significantly decreased while glutamine was increased in the cerebral cortex and brain stem of hepatectomized rats. Alanine was significantly raised in the cortex of hepatectomized rats. GABA was unchanged in both regions.

Plasma amino acid levels did not differ significantly, however, there was a low number of rats in each group. Some amino acids, i.e. phenylalanine, tyrosine and methionine tended to increase while the BCAA tended to decrease.

The present study demonstrates that rapid changes develop in the concentrations of glutamine, glutamate, aspartate and alanine in the frontoparietal part of the cerebral cortex 4 hours after hepatectomy. Similar changes were also prevalent in the brain stem. It should be stressed that these occurred without any tendency to disruption of the brain energy state. On the contrary, there was a small increase in the ATP concentration after hepatectomy. Furthermore, no significant decrease in the sum of the phosphocreatine and creatine concentrations occurred. This is of interest since previous conclusions that ammonia intoxication interferes with energy metabolism in the brain stem are partly based on observed decreases in phosphocreatine concentrations (8). In shunted rats exposed to ammonia intoxication with similar changes in the levels of ammonia-related amino acids, the energy state was unchanged in precomatose animals. Decreases of the ATP concentrations and the ATP/ADP ratios occurred, however, one hour after the ammonia load in comatose rats (11). The similarities between the metabolic impact of hepatectomy and advanced hyperammonemia after porta-caval shunt and the dissimilarities with respect to the energy phosphates and the energy state may partly be explained by differences in ammonia accumulation and time course. Moreover, total hepatectomy is also a much more complex model that causes many metabolic disturbances. The minor manifestations of cerebral dysfunction in hepatectomized rats can obviously not be attributed to energy failure at least not in the early phase of the hepatectomized state. Thus, these early precomatose symptoms must be due to other mechanisms and one possibility is interference with the putative transmitters of the glutamate group, as suggested by Holmin (10) and Hindfelt et al (11).

It is of interest that the present study in hepatectomized rats demonstrates clear-cut changes in the concentration of glutamine, glutamate and aspartate in brain tissue, but no significant changes in plasma amino acid concentrations compared with the shunted control rats. Increases in brain/plasma concentration ratios of the neutral amino acids after porta-caval anastomoses and after repeated injections of ammonium acetate in rat (47, 60) have been suggested to play a role in the pathogenesis of hepatic encephalopathy. However, in the present study brain concentrations of large neutral amino acids were not studied. The changes in ammonia-related amino acids in brain tissue was the main finding. The early precomatose symptoms after hepatectomy may therefore not be ascribed to energy failure nor to plasma amino acid imbalance.

Combined or individual administration of BCAA following total hepatectomy in the rat: effects on amino acids and indoleamines in the brain (III)

Administration of BCAA to patients and dogs with liver failure and encephalopathy tended to normalize plasma amino acid levels (53, 69, 86, 88). In most studies, a combined infusion of various concentrations of valine, leucine and isoleucine together with other amino acids has been used. In the study of Riederer et al (75)

administration, of a solution containing 5% valine to patients with hepatic coma resulted in improvement of encephalopathy and decreased levels of tryptophan, serotonin and 5-HIAA in the reticular formation. Ericsson et al (131) and Hagenfeldt et al (132) studied the effect of individual branched chain amino acids on blood amino acid levels in healthy subjects. These studies showed that infusion of leucine alone resulted in significant decreases in 13 of 19 other amino acids including methionine, isoleucine, valine, tyrosine, and phenylalanine. Infusion of valine resulted in lower concentrations of tyrosine only, and isoleucine administration did not change the concentrations of any other amino acid. Previous experiments in normal rats have also shown that a diet containing 3% or 8% leucine resulted in lower levels of serotonin in the brain (133). These reported effects of leucine alone to decrease other amino acids in plasma in normal subjects and of valine to reduce indoleamines in brain made it interesting to study the effects of raising the plasma concentrations of valine, leucine and isoleucine either singly or in combination, on the concentrations of plasma and brain amino acids and on brain indoleamine metabolism in rats after hepatectomy.

Hepatectomy and glucose infusion (group A) resulted in significant increases in plasma of several amino acids, i.a. histidine (170%), tyrosine (196%), methionine (210%), phenylalanine (179%), threonine (38%), alanine (53%), glutamine (185%), tyrosine (386%) and decreases of isoleucine (74%), leucine (59%) and valine (55%) compared to control group H.

In the brain, hepatectomy and glucose infusion resulted in higher concentrations of i.a. histidine (376%), lysine (55%), methionine (400%), phenylalanine (550%), glutamine (364%), tyrosine (854%), tryptophan (226%), and 5-HIAA (100%) while concentration of glutamate was decreased (21%) compared to control group H.

Single infusion of valine, leucine and isoleucine resulted in a 22-fold, 9-fold and 27-fold rise of these amino acids in plasma, respectively. The comparatively low increase in the level of leucine may be explained by the almost twice as high rate of entry for leucine compared with valine and isoleucine in rat muscle (134). Such an infusion did not result in any decrease of the other BCAA in plasma, in contrast to reports on healthy humans (131, 132).

Combined infusion of 0.1 mol/l BCAA resulted in non-significant rises of isoleucine (7-fold), leucine (3-fold) and valine (3-fold). Administration of 0.3 mol/l BCAA resulted in significant rises of isoleucine (56-fold), leucine (17-fold) and valine (16-fold).

Simultaneous infusion of glucose and BCAA (Table 1) after hepatectomy, compared with glucose alone, decreased the accumulation in plasma of other essential amino acids and tyrosine as follows: infusion of 0.1 mol/l (combined) BCAA resulted in a reduction in 4 of those 7 amino acids and infusion of 0.3 mol/l BCAA in 6.

Furthermore, the concentrations of methionine, phenylalanine, threonine, and tyrosine were significantly lower after infusion of 0.3 mol/l than of 0.1 mol/l (combined) BCAA.

Infusion of 0.1 mol/l valine resulted in lower concentrations in 3 of these 7 amino acids, infusion of 0.1 mol/l leucine alone only decreased methionine accumulation significantly, and infusion of 0.1 mol/l isoleucine did not lower any of these amino acids in plasma.

Table 1

Significant changes (%) of some amino acids in plasma after various infusions of BCAA and glucose compared to glucose alone in hepatectomized rats.

<u>Treatment (n)</u>	Valine (7) 0.1 M	Leucine (7) 0.1 M	Isoleucine (7) 0.1 M	BCAA (6) 0.1 M	BCAA (7) 0.3 M
<u>Amino acids</u>					
Histidine			+24		
Lysine	-25				-30
Methionine	-37	-31		-53	-84
Phenylalanine			+16	-33	-66
Threonine					-58
Tryptophan	-36			-48	-62
Tyrosine				-19	-41
Alanine			+138		+199
Glutamine		+35	+51		+64

Previous *in vitro* studies in which BCAA were incubated with the diaphragm of rats demonstrated that leucine increased protein synthesis and decreased protein degradation: these effects were also shown for isoleucine and valine together, but not individually (100, 101). In the present study, combined infusion of BCAA in hepatectomized animals seemed to affect the rise in essential amino acids in plasma more than infusion of individual BCAA. This may suggest that all three BCAA are more effective than any one in affecting protein metabolism *in vivo*. However, it is not possible

to conclude whether infusion of BCAA affected protein metabolism by stimulating protein synthesis or by inhibiting proteolysis. Other mechanisms may also be present.

Concentrations of alanine in plasma were significantly higher after infusion of 0.1 mol/l isoleucine and 0.3 mol/l BCAA than after glucose infusion alone. Glutamine concentrations in plasma were higher than in glucose-infused, hepatectomized rats after infusion of 0.1 mol/l leucine, 0.1 mol/l isoleucine and 0.3 mol/l BCAA. These findings are in accordance with *in vitro* studies which have demonstrated that the synthesis of alanine and glutamine by muscle is stimulated by incubating the muscle with each of the BCAA (135). Leucine has also been reported to stimulate the release of alanine and glutamine from isolated perfused rat hind-quarters and from the human forearm (136, 104). Transamination of the BCAA occurs in muscle with α -ketoglutarate to form glutamate which in turn may either transaminate with pyruvate to form alanine or incorporate free ammonia to form glutamine.

In the brain (Table 2), all infusions of BCAA either singly or in combination, reduced concentrations of histidine, methionine, phenylalanine, and tyrosine. These reductions were greater for 0.3 mol/l BCAA than in all the other groups. The concentrations of tryptophan in the brain were reduced significantly more by 0.3 mol/l BCAA than by 0.1 mol/l BCAA. Infusion of leucine, but not of valine and isoleucine alone also reduced brain tryptophan significantly. The level of serotonin in brain, however, was only reduced by 0.3 mol/l BCAA. Compared with hepatectomized rats receiving glucose alone, all infusions of BCAA, either singly or in combination, resulted in lower brain levels of the principal metabolite of serotonin, 5-HIAA, which may indicate reduced turnover of serotonin after treatment.

Infusion of individual BCAA resulted in significant reductions of several LNAA in the brain, in spite of unchanged concentrations of most of these amino acids in plasma. Thus, by infusing BCAA, either singly or in combination, it was possible to raise the competition among LNAA for blood-brain transport. These results demonstrate that the BCAA may lower brain serotonin concentrations by affecting both peripheral protein metabolism and blood-brain amino acid transport.

In the present study it was also shown that the concentration of glutamate was decreased in the brain of glucose-treated, hepatectomized rats and that infusion of BCAA, individually or combined, prevented the decrease of glutamate in brain.

Table 2

Significant changes (%) of some amino acids and indoleamines in brain after various infusions of BCAA and glucose compared to glucose alone in hepatectomized rats.

<u>Treatment (n)</u>	Valine (7) 0.1 M	Leucine (7) 0.1 M	Isoleucine (7) 0.1 M	BCAA (6) 0.1 M	BCAA (7) 0.3 M
<u>Amino acids</u>					
Isoleucine			+1464	+386	+714
Leucine		+147		+113	+207
Valine	+1231				+211
Histidine	-20	-42	-39	-26	-74
Lysine					
Methionine	-54	-60	-57	-63	95
Phenylalanine	-37	-48	-40	-45	-92
Threonine					-44
Tyrosine	-33	-45	-44	-30	-83
Alanine					
Glutamate	+20		+21		+23
Glutamine					-30
<u>Indoleamines</u>					
Tryptophan		-27		-55	-88
Serotonin					-31
5-HIAA	-33	-42	-36	-30	-60

Amino acids in plasma, amino acids and catecholamines in the brain
6 and 18 hours after total hepatectomy - effects of branched chain
amino acids (IV)

High levels of phenylalanine and tyrosine have been suggested to alter catecholamine synthesis in brain resulting in lower levels of dopamine and norepinephrine, and increased concentrations of "false neurotransmitters" (63). In the present study the effect of BCAA on decreased levels of catecholamines in the brain after hepatectomy was examined.

Hepatectomized and control rats were sacrificed at 6 and 18 hours after surgery. Before sacrifice 6 hours after hepatectomy, all the rats were comparatively alert. In the hepatectomized group infused with glucose alone, 9 of 14 rats survived 18 hours and 8 of these 9 rats were in coma (unresponsive to tail-pinch) at sacrifice. In the BCAA treated group 9 of 15 rats survived 18 hours and 4 of these 9 rats were in coma at sacrifice. The difference in incidence of coma was not statistically significant (χ^2 test). Since physiological variables were not controlled it cannot be excluded that hypoxaemia, arterial hypotension, changes in carbon dioxide tension or other factors could contribute to coma and death. Hypoglycemia was prevented by glucose infusion with no significant differences of glucose concentration in plasma between the groups. The MANOVA results indicated overall significant differences ($p < 0.001$) among amino acids and catecholamines in both plasma and brain. Significant ($p < 0.05$ at least) univariate 4 x 2 factorial ANOVAS in both plasma and brain were obtained for all amino acids and catecholamines with these exceptions: brain aspartate for time, brain serine for time, plasma glutamate for treatment x time, brain glutamate for treatment and for treatment x time, and all three BCAA for time.

After hepatectomy and glucose infusion alone, there was a progressive rise of many amino acids. In plasma, concentrations of aspartate, threonine, serine, glutamine, alanine, methionine, tyrosine, phenylalanine, lysine, histidine and arginine were significantly higher at 18 hours when compared with 6 hours after hepatectomy (Figs 5 and 6). In the brain, threonine, glutamine, glycine, alanine, methionine, tyrosine, phenylalanine, lysine, histidine and arginine were higher at 18 hours (Fig 7). In contrast to the other experiments in the present study, there was no significant fall of glutamate after hepatectomy and glucose infusion.

Simultaneous infusion of glucose and BCAA to hepatectomized rats resulted in the following changes compared with glucose infusion alone; in plasma, concentrations of threonine, methionine, tyrosine, phenylalanine, lysine and histidine were significantly lower both at 6 and 18 hours in the BCAA treated group while glutamine, alanine and the BCAA were higher than in the glucose treated group; at 18 hours only, concentrations of aspartate and arginine were also higher. In the brain, concentrations of threonine, methionine,

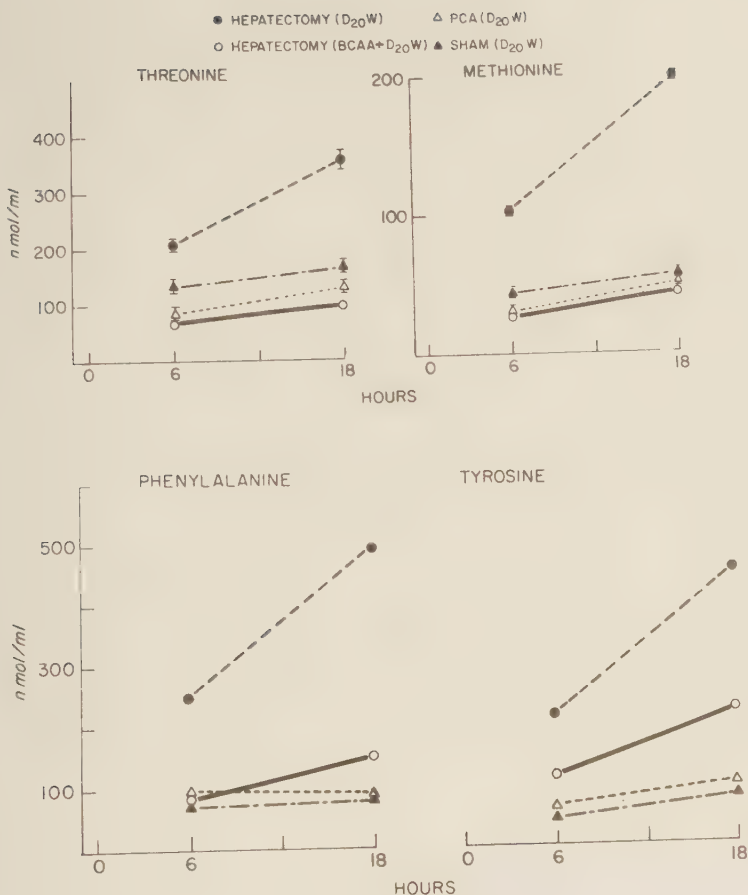


Fig 5. Plasma concentrations (means $\pm$ SEM) of some amino acids after 6 and 18 hours in hepatectomized or control rats. Hepatectomized animals were infused with D₂₀W (20% glucose solution) or D₂₀W + branched chain amino acids (BCAA). Control rats were infused with D₂₀W. PCA = rats operated with division of the inferior vena cava, porta-caval anastomosis and sham-hepatectomy. SHAM = rats operated with division of the inferior vena cava, sham-porta-caval anastomosis and sham-hepatectomy.

Lines are drawn only to facilitate inspection of data in this and in the following figures.

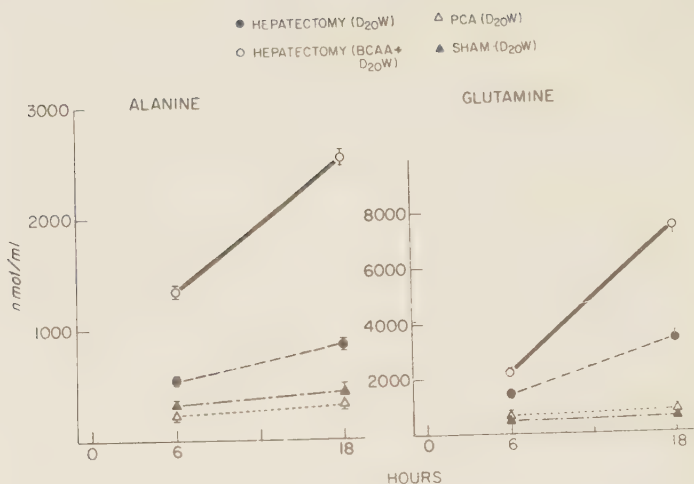


Fig 6. Plasma concentrations (means $\pm$ SEM) of alanine and glutamine after 6 and 18 hours in hepatectomized or control rats. Hepatectomized animals were infused with D₂₀W (20% glucose solution) or D₂₀W + branched chain amino acids (BCAA). Control rats were infused with D₂₀W. PCA = rats operated with division of the inferior vena cava, porta-caval anastomosis and sham-hepatectomy. SHAM = rats operated with division of the inferior vena cava, sham-porta-caval anastomosis and sham-hepatectomy.

tyrosine, phenylalanine, lysine and histidine were lower in the BCAA treated animals both at 6 and 18 hours, and at 18 hours only alanine was lower. At both 6 and 18 hours, the BCAA were higher and at 18 hours glycine and arginine were higher in the BCAA treated groups. Concentrations of tryptophan in plasma and brain were lower at both 6 and 18 hours after infusion of BCAA and glucose than after glucose infusion alone.

The present results demonstrate that also 18 hours after hepatectomy infusion of BCAA reduced the accumulation of threonine, methionine, tyrosine, phenylalanine and histidine in the brain. This effect of BCAA appeared to be a result of two separate phenomena including reduced accumulation of these neutral amino acids in the circulation as discussed previously and competition for blood-brain transport via the neutral amino acid transport system. Thus, the difference between the concentrations of those five amino acids in the two hepatectomized groups was much greater in the brain than in plasma. By calculating brain/plasma concentrations ratios it also appears that the ratio is lower in the BCAA treated group for

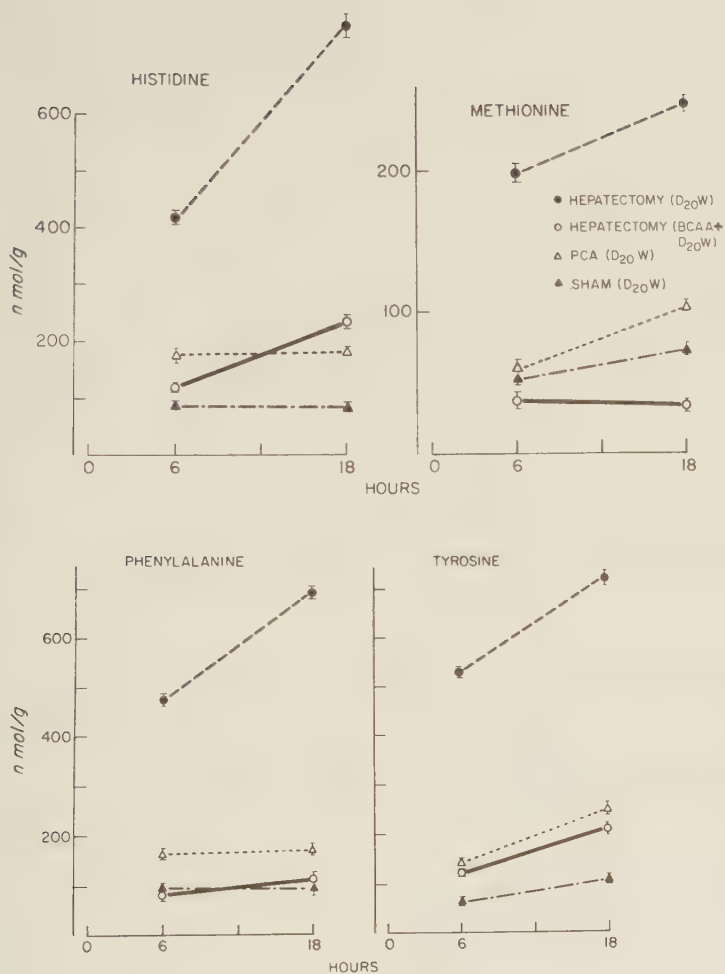


Fig 7. Brain concentrations (means $\pm$ SEM) of some amino acids after 6 and 18 hours in hepatectomized or control rats. Hepatectomized animals were infused with D₂₀W (20% glucose solution) or D₂₀W + branched chain amino acids (BCAA). Control rats were infused with D₂₀W. PCA = rats operated with division of the inferior vena cava, porta-caval anastomosis and sham-hepatectomy. SHAM = rats operated with division of the inferior vena cava, sham-porta-caval anastomosis and sham-hepatectomy.

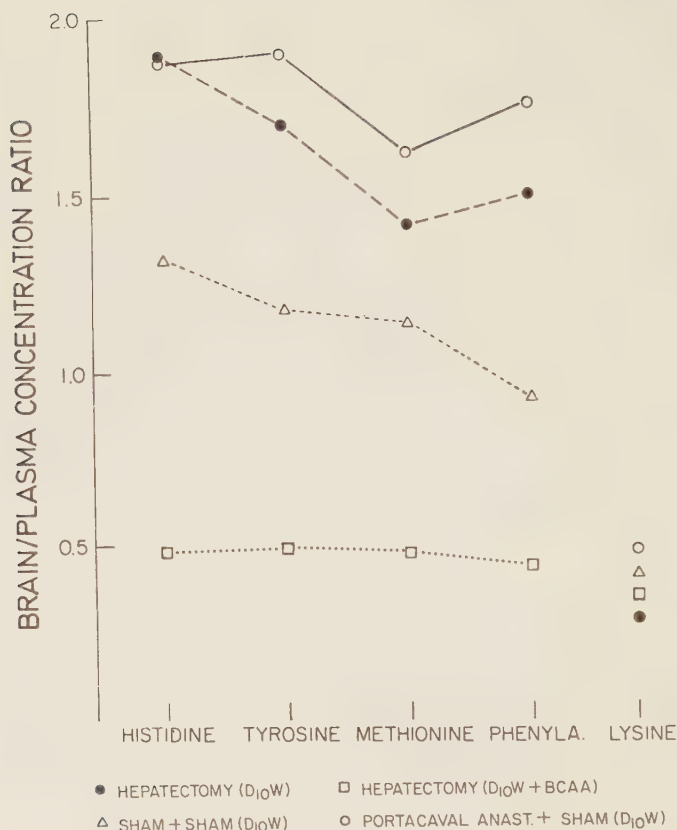


Fig 8. Brain/plasma concentration ratio of the neutral amino acids histidine, tyrosine, methionine and phenylalanine and the basic amino acid lysine in hepatectomized and control rats. D₁₀W = 10% glucose solution. BCAA = branched chain amino acids. All rats underwent division of the inferior vena cava.

some neutral amino acids but not for the basic amino acid lysine (Fig 8, data from paper V). These differences may suggest that high concentrations of BCAA in the blood reduced the uptake of other LNAA by the brain when competing for transport across the blood-brain barrier. However, Livingstone et al. (137) showed that physical breakdown of the blood-brain barrier occurs late in acute hepatic failure in hepatectomized rats. The authors demonstrated that the barrier had become permeable to D-sucrose, inulin, L-glucose and trypan blue. The present study shows that with respect

to competition for neutral amino acid transport the blood-brain barrier seems to be functional at 18 hours after hepatectomy.

Six hours after surgery, norepinephrine levels were unchanged in both hepatectomized groups compared to the control groups. Eighteen hours after hepatectomy, regardless of treatment, norepinephrine levels were reduced while dopamine levels were unchanged. Thus, lower levels of norepinephrine were obtained only at 18 hours, when 12 of 18 rats were in coma, and rats in coma had significantly less norepinephrine in brain than did non-comatose rats. Infusion of BCAA to hepatectomized rats resulted in levels of phenylalanine and tyrosine lower than or similar to those obtained in the controls with porta-caval shunt, in which brain norepinephrine was near normal levels. The present results are in agreement with previous studies of hepatectomized rats (71) and suggest that high concentrations of phenylalanine and tyrosine do not appear to be the only cause of decreased norepinephrine levels after hepatectomy.

Regional analysis of norepinephrine and dopamine in the brain
18 hours after hepatectomy and infusion of BCAA (V).

Since levels of norepinephrine were slightly higher in the BCAA-treated group in paper IV without achieving statistical significance, a study of catecholamines in eight different regions of the brain was performed. The infusion rate was increased and the concentration of glucose in the solution dropped to 10%.

Hepatectomy and glucose infusion alone (group I) resulted again in elevations of the concentrations of several amino acids including phenylalanine and tyrosine in plasma and the brain (Fig 9). Dopamine levels in group I were significantly lower in striatum and higher in pons-medulla than in Group III or IV (Fig 10). Concentrations of norepinephrine were lower in all brain regions of Group I than in those of Group III and IV except in the striatum where they remained unchanged. These results are in agreement with those obtained by Tyce and Owen (70) in hepatectomized rats except in the striatum in which these investigators observed decreased norepinephrine and unchanged dopamine levels. In further studies, Tyce and Owen (138) reported that administration of ^{14}C -dopa to hepatectomized rats resulted in higher brain concentrations of dihydroxy-phenylacetic acid, a metabolite of dopamine, than in the control rats, suggesting an impairment of vesicular uptake or retention of dopamine or an inhibition of β -hydroxylation. No significant differences in dopamine or norepinephrine levels were observed between the control groups III and IV in any region, although concentrations of phenylalanine and tyrosine in the brain of rats with porta-caval anastomosis were about twice the corresponding levels in the control group IV. These results are partly in agreement with Bucci et al. (139) who demonstrated increased levels of norepinephrine in some regions of the brain in rats with porta-caval shunt.

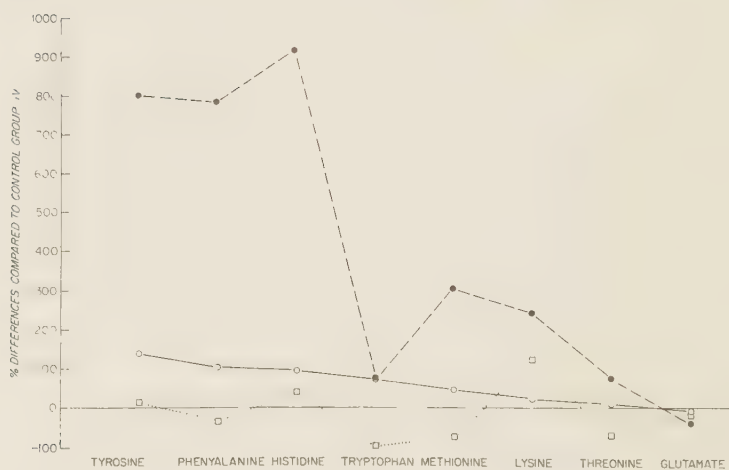
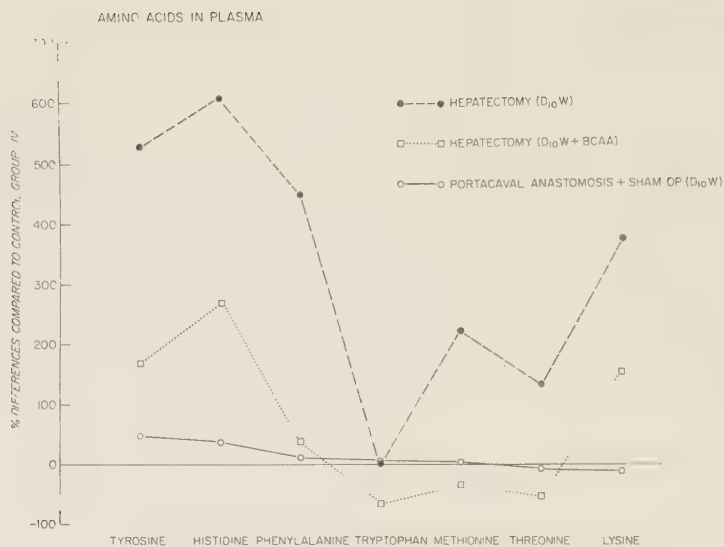


Fig 9. Amino acids in plasma and cerebral cortex after 18 hours in hepatectomized or control rats. D₁₀W = 10% glucose solution. BCAA = branched chain amino acids. All rats underwent division of the inferior vena cava. Animals in group IV also underwent sham-portacaval anastomosis and sham-hepatectomy.

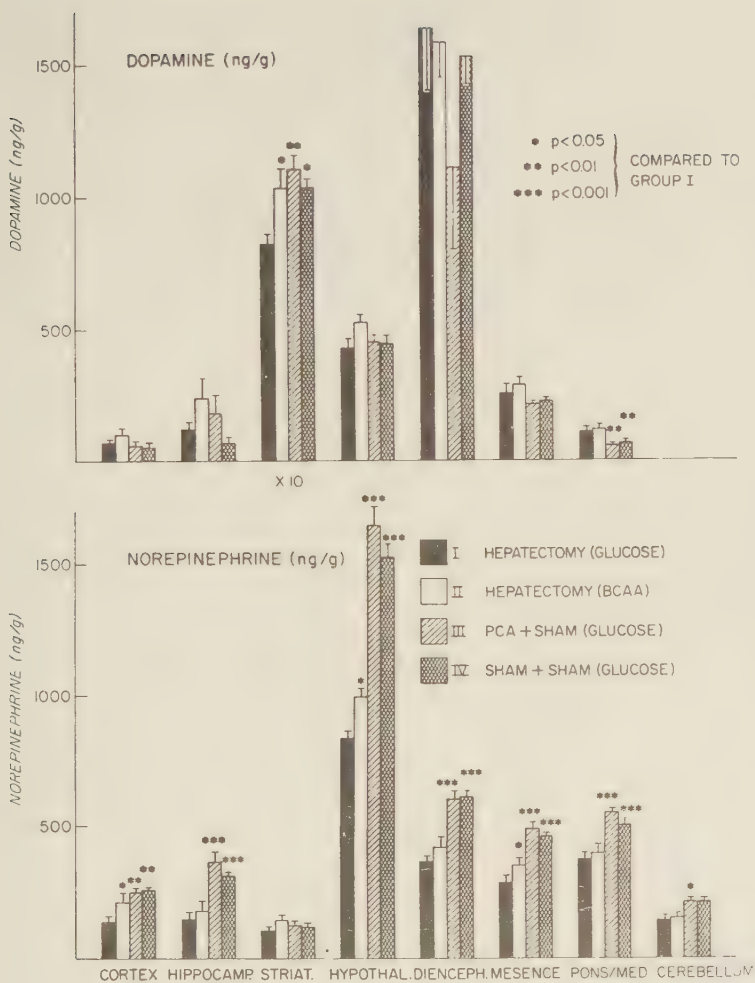


Fig 10. Dopamine and norepinephrine concentrations (means $\pm$ SEM) in different brain regions after 18 hours in hepatectomized or control rats. PCA + SHAM = porta-caval anastomosis + sham-hepatectomy. SHAM + SHAM = sham-porta-caval anastomosis + sham-hepatectomy. All rats also underwent division of the inferior vena cava. Cortex = cerebral cortex; Hippocamp = hippocampus; Striat = striatum; Hypothal = hypothalamus; Dienceph = diencephalon; Mesence = mesencephalon; Pons/Med = pons and medulla.

Infusion of BCAA resulted again in lower levels of tyrosine, phenylalanine, histidine, tryptophan, methionine and lysine in plasma and cerebral cortex (Fig 10) and the levels of phenylalanine and tyrosine in cortex were similar to those obtained in the controls with division of the inferior vena cava and sham operations (Group IV). Thus, infusion of the BCAA provided a means of dissociating the absence of liver function from high brain levels of many neutral amino acids. Infusion of BCAA (Group II) resulted in higher levels of dopamine in the striatum and in higher levels of norepinephrine in the hypothalamus, cortex and mesencephalon compared with Group I. However, in the hypothalamus and mesencephalon the concentrations of norepinephrine were still significantly lower than in the controls. The increases in norepinephrine in the striatum, hippocampus and remaining diencephalon were not statistically significant. Thus, also after infusion of BCAA the concentrations of norepinephrine in most brain regions were low. If it is assumed that amino acid levels including phenylalanine and tyrosine in cortex are close to the amino acid levels in other brain regions as has been reported for rats with porta-caval shunt by Bucci et al. (139), then it is unlikely that high levels of tyrosine or phenylalanine in the brain are the only cause of low brain norepinephrine levels after hepatectomy. It was also noted that considering all groups together, no significant correlation was observed between the ratio of phenylalanine to tyrosine in the cerebral cortex and norepinephrine levels in any region. Nevertheless, BCAA infusion after hepatectomy was associated with higher brain levels of norepinephrine in the hypothalamus, cortex and mesencephalon and higher dopamine levels in the striatum as compared with hepatectomized rats receiving glucose. It is not possible from the present data to explain the low levels of norepinephrine after hepatectomy. The decreased concentrations of norepinephrine after hepatectomy might be the result of enhanced release and breakdown of the transmitter or of a blocking of the β -hydroxylation of dopamine to norepinephrine. It may be assumed that by infusing BCAA and reducing plasma and brain levels of phenylalanine, tyrosine and tryptophan, concentrations of biogenic amines ("false neurotransmitters") would be reduced. These amines have been suggested to replace norepinephrine from storage sites and tyramine and β -phenylethylamine may compete with dopamine for β -hydroxylation (63). Thus BCAA infusion may reduce these effects on decreased norepinephrine levels by false neurotransmitters. It is, however, impossible to eliminate such effects since some amines may be formed in the periphery and penetrate a disrupted, some of them even a non-disrupted, blood-brain barrier (140).

An unanticipated inverse correlation was observed between $\log_{10}$ of the brain alanine concentration and brain norepinephrine levels in paper IV. Also in paper V an inverse linear relationship was observed between norepinephrine concentrations and the $\log_{10}$ of the alanine concentration in cortex. However, it is unlikely that high concentrations of alanine interfered with inhibited synthesis or stimulated degradation of norepinephrine.

Administration of BCAA resulted in other changes of amino acids of which some may be of interest in hepatic encephalopathy. Decreases of glutamate levels were partly prevented in the cerebral cortex while decreased levels of aspartate were not affected. Tryptophan concentrations were greatly reduced and could not be detected in the BCAA treated group.

The present experiments show that administration of BCAA makes possible the manipulation of neutral amino acids and certain neurotransmitters. Survival rates and the number of rats in coma 18 hours after hepatectomy were, however, not significantly influenced by the addition of BCAA to the glucose infusion used.

SUMMARY AND CONCLUSIONS

In the present study a reliable microsurgical technique for total hepatectomy in the rat was developed and some biochemical changes associated with hepatic encephalopathy and coma were examined. The effects of infusion of BCAA on amino acid and neurotransmitter levels after infusion of BCAA in hepatectomized rats were also investigated.

Several metabolic disturbances have been proposed to play a role in the etiology of hepatic encephalopathy. The deranged ammonia metabolism interferes with brain energy metabolism, and hepatic coma has been suggested to result from power failure. Other manifestations of the affected ammonia metabolism in the brain, i.e. the decreased concentrations of the putative excitatory neurotransmitters glutamate and aspartate, and increased levels of glutamine may be important. Increased levels of the inhibitory neurotransmitter GABA in brain tissue have been suggested to be of significance. Increased concentrations of aromatic amino acids in the brain have been proposed to result in depletion of norepinephrine and dopamine and increased synthesis of "false neurotransmitters" which may displace norepinephrine from storage sites. Elevated levels of tryptophan in brain may result in increased serotonin levels and thus further alter neurotransmission.

Traditional treatment of hepatic encephalopathy aims at lowering ammonia levels in blood. Recently, administration of BCAA has been introduced in order to correct plasma amino acid imbalance and normalize the suggested disturbances in neurotransmission.

- I A technique was developed to obtain a safe and reproducible model for the study of metabolic changes occurring after hepatectomy. The complete removal of the liver was realized five weeks after division of the inferior vena cava just below the liver and one week after construction of a modified porta-caval shunt. The technique implies the use of a microsurgical technique and preservation of the flow through the gastroduodenal (pancreatico-duodenal) vein. The developed model made it possible to perform studies in 65% of warm rats (35-37°C) 18 hours after hepatectomy. Most other studies of warm hepatectomized rats have been performed within 6 hours after hepatectomy, and investigations at a later time after the removal of the liver have usually been carried out in cold rats (28-29°C).
- II Hepatectomy did not disrupt the balance between production and utilization of energy in the cerebral cortex and brainstem 4 hours after surgery. At the same time, no significant changes in plasma amino acid levels occurred. On the other hand, the brain levels of glutamate, aspartate and glutamine were changed in the expected directions when compared with shunted

control rats. The concentrations of GABA in the brain did not differ between the hepatectomized and the control rats.

- III Hepatectomized glucose-infused rats had higher plasma concentrations of several amino acids including histidine, tyrosine, methionine, phenylalanine, threonine, alanine and glutamine and lower BCAA concentrations than sham-hepatectomized controls when studied 6 hours post-hepatectomy. In the brain, concentrations of glutamine, the neutral amino acids (except the BCAA) and 5-HIAA were markedly elevated.

When all three BCAA, isoleucine, valine and leucine, were added to the glucose infusion in the hepatectomized rats significant reductions in plasma concentrations of other essential amino acids and tyrosine were obtained. These reductions were more pronounced for 0.3 mol/l BCAA than for 0.1 mol/l BCAA. Infusion of 0.1 mol/l isoleucine did not significantly reduce any essential amino acid in plasma while 0.1 mol/l valine reduced lysine, methionine and tryptophan and 0.1 mol/l leucine reduced only methionine. Infusion of BCAA resulted in increased plasma concentrations of glutamine and alanine.

In the brain, infusions of BCAA, either singly or in combination, reduced the levels of other neutral amino acids. Thus, competition for transport at the blood-brain barrier was probably present 6 hours after hepatectomy. Infusion of BCAA in combination or leucine alone gave rise to a decreased tryptophan concentration. Infusion of BCAA, singly or in combination, reduced 5-HIAA while only 0.3 mol/l BCAA reduced serotonin. Infusion of 0.1 mol/l valine, 0.1 mol/l isoleucine or 0.3 mol/l BCAA partly prevented the decrease in brain concentration of glutamate. This effect on the glutamate level was also demonstrated in the cerebral cortex 18 hours post-hepatectomy (V).

- IV Hepatectomy followed by glucose infusion i.v. resulted in a progressive increase in several amino acids in plasma and brain 6 and 18 hours after the hepatectomy. In the brain of rats with a porta-caval anastomosis or 6 hours post-hepatectomy the significant increases in aromatic amino acids were associated with unchanged levels of norepinephrine. The norepinephrine levels were lower than in the control rats 18 hours post-hepatectomy.

The addition of BCAA to glucose-infused hepatectomized rats resulted in levels of phenylalanine and tyrosine in the brain that were lower or similar to those of the controls with porta-caval shunt at 18 hours after surgery. However, in the control rats with a porta-caval anastomosis, norepinephrine

levels were near normal and in the BCAA treated animals a decreased norepinephrine level was obtained. Comatose rats had lower norepinephrine in the brain than non-comatose.

Administration of BCAA did not increase the survival rate 18 hours after hepatectomy.

- V Regional analysis of catecholamines in the brain 18 hours after hepatectomy followed by i.v. glucose infusion showed low levels of norepinephrine in 7 of 8 regions. The level of dopamine was low in striatum and high in pons-medulla. Increased infusion rate of BCAA resulted in levels of tyrosine and phenylalanine in cerebral cortex that were similar to those of control animals without a porta-caval shunt. In these BCAA-treated hepatectomized rats, norepinephrine was significantly increased in cortex, hypothalamus and mesencephalon, and dopamine levels were significantly increased in striatum when compared with hepatectomized glucose-infused rats. However, the levels of norepinephrine in hypothalamus and mesencephalon were still significantly lower than in the controls. These results (IV, V) suggest that high concentrations of tyrosine and phenylalanine do not appear to be the only cause of low norepinephrine levels after hepatectomy. It is also shown that with respect to competition between large neutral amino acids the blood-brain barrier seems to be in function 18 hours after hepatectomy.

The present studies confirm previously demonstrated metabolic disturbances in plasma and brain tissue of rats subjected to total hepatectomy. Since the developed microsurgical model for complete removal of the liver allowed studies in animals with a near-normal body temperature, some new information about the late phase of the hepatectomized state was obtained.

In the early phase after hepatectomy clear-cut changes in the ammonia-related amino acids of the brain were present simultaneously with a preserved brain energy state and essentially unchanged plasma amino acid levels. Some of the ammonia-related amino acids probably act more or less as potent neurotransmitters and the changed levels of these amino acids may play a role for altered neurotransmission. The significance of the demonstrated changes in brain metabolic state for the pathogenesis in hepatic encephalopathy was not evaluated.

The addition of BCAA to hepatectomized rats reduced the accumulation of several other plasma and brain amino acids and affected the levels of certain neurotransmitters. However, further studies are required to clarify the mechanism by which BCAA affect the concentrations of other neutral amino acids in the periphery and CNS. The data obtained in the present study should stimulate further investigation of the effects of BCAA in the treatment of patients with hepatic encephalopathy.

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